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Belibasakis, Georgios N ; Bostanci, Nagihan

Abstract: BACKGROUND AND OBJECTIVES: The receptor activator of NF- κ B ligand-osteoprotegerin (RANKL-OPG) bi-molecular system is the "bottle-neck" regulator of osteoclastogenesis and bone resorption, both in physiological and pathological conditions. This review aims to elaborate the current knowledge on RANKL and OPG in periodontal disease, and to evaluate their diagnostic and prognostic potential as biomarkers of the disease. MATERIALS AND METHODS: To pursue this aim, electronic and manual searches were performed for identifying clinical and in vivo studies on RANKL and OPG in gingival tissue, gingival crevicular fluid, saliva and blood. Smoking and diabetes mellitus were also considered for their potential effects. RESULTS: Papers fulfilling the inclusion criteria demonstrate that RANKL is up-regulated, whereas OPG is down-regulated in periodontitis, compared to periodontal health, resulting in an increased RANKL/OPG ratio. This ratio is further up-regulated in smokers and diabetics, and is not affected by conventional periodontal treatment. CONCLUSIONS: The increased RANKL/OPG ratio may serve as a biomarker that denotes the occurrence of periodontitis, but may not necessarily predict on-going disease activity. Its steadily elevated levels post treatment may indicate that the molecular mechanisms of bone resorption are still active, holding an imminent risk for relapse of the disease. Additional adjunct treatment modalities that would "switch-off" the RANKL/OPG ratio may therefore be required.

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The RANKL-OPG system in Clinical Periodontology

Georgios N. Belibasakis *¹ and Nagihan Bostanci ²

¹ Oral Microbiology and Immunology, ² Oral Translational Research,
Institute of Oral Biology, Center of Dental Medicine, Faculty of
Medicine, University of Zürich, Switzerland

* Author for correspondence:

Dr. Georgios N. Belibasakis

Institute of Oral Biology

Center of Dental Medicine

University of Zürich

Plattenstrasse 11

8032 Zürich

Switzerland

e-mail: george.belibasakis@zzm.uzh.ch

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Running title: RANKL-OPG in Clinical Periodontology

Abstract

Background and Objectives: The RANKL-OPG bi-molecular system is the “bottle-neck” regulator of osteoclastogenesis and bone resorption, both in physiological and pathological conditions. This review aims to elaborate the current knowledge on RANKL and OPG in periodontal disease, and evaluate their diagnostic and prognostic potential as biomarkers of the disease.

Materials and Methods: To pursue this aim, electronic and manual searches were performed for identifying clinical and *in vivo* studies on RANKL and OPG in gingival tissue, gingival crevicular fluid, saliva and blood. Smoking and diabetes mellitus were also considered for their potential effects.

Results: Papers fulfilling the inclusion criteria demonstrate that RANKL is up-regulated, whereas OPG is down-regulated in periodontitis, compared to periodontal health, resulting in an increased RANKL/OPG ratio. This ratio is further up-regulated in smokers and diabetics, and is not affected by conventional periodontal treatment.

Conclusions: The increased RANKL/OPG ratio may serve as a biomarker that denotes the occurrence of periodontitis, but may not necessarily predict on-going disease activity. Its steadily elevated levels post-treatment may indicate that the molecular mechanisms of bone resorption are still active, holding an imminent risk for relapse of the disease. Additional adjunct treatment modalities that would “switch-off” the RANKL/OPG ratio may therefore be required.

Conflict of interests and source of funding statement

The authors state that they have no conflict of interest. The study was self-funded by the authors and their Institution.

Introduction

Bone is a dynamic tissue hard tissue that is subjected to continuous remodelling, in order to meet the functional adaptation needs. The remodelling process is characterised by coupling of resorption of the bone matrix by osteoclasts, and its re-formation by osteoblasts. The alveolar bone of the periodontium is no exception to this process. Remodelling of the alveolar bone can occur in physiological situations, such as occlusal forces, tooth eruption, clinical interventions, such as orthodontic tooth movement, and pathological conditions, such as periodontitis and periapical pathoses. Under physiological conditions, a dynamic balance is established between bone formation and resorption. However, if the balance switches towards enhanced bone resorption, then bone destructive pathology will occur (Lerner 2004).

The long-sought molecular mechanisms behind the cell-to-cell interaction that regulate bone resorption were elucidated in the late 1990s. Receptor Activator of Nuclear Factor- κ B Ligand (RANKL), a member of the Tumor Necrosis Factor (TNF) ligand superfamily, was identified as a cell membrane-bound factor responsible for stimulation of osteoclast differentiation and bone resorption (Kong et al. 1999; Lacey et al. 1998). RANKL is produced as a membrane-bound or secreted ligand by osteoblasts, fibroblasts, or activated T- and B-cells. Of note, three isoforms of RANKL have been identified (Ikeda et al. 2001), which can potentially multimerize (Ikeda et al. 2003): RANKL1 possesses an intracellular, transmembrane and extracellular domain, RANKL2 has a shorter intracellular domain, and RANKL3 lacks both the intracellular and transmembrane domain, and is thought to act as soluble ligand. These three different variants may have different roles or potencies in the regulation of osteoclastogenesis, with RANKL1 being the predominant inducer, whereas RANKL3 a potential attenuator (Suzuki et al. 2004).

By activating its cognate RANK receptor on the surface of pre-osteoclasts, it triggers their fusion and differentiation into mature osteoclasts, thus activating bone resorption (Teitelbaum et al. 2003). The action of RANKL can be blocked by its soluble decoy receptor osteoprotegerin (OPG), which is a member of the TNF receptor superfamily, with structural homology to RANK (Simonet et al. 1997). By binding to RANKL, OPG prevents its further interaction with RANK, and subsequently all the down-stream molecular events that lead to osteoclast differentiation and bone resorption. A simplified diagrammatic representation of the RANKL-RANK-OPG interplay is provided in the Figure. The production of RANKL and OPG by various cells types is controlled by systemic and local stimuli, including hormones, inflammatory mediators and bacterial products (Lerner 2006; Liu et al. 2010).

Increased RANKL or decreased OPG local expression can cause bone resorption at various sites of the human skeleton. Conversely, decreased RANKL or increased OPG expression could result in enhanced bone formation, leading to osteopetrotic conditions. The involvement of the RANKL-OPG system is well established in the pathogenesis of diseases of bone and mineral metabolism, such as rheumatoid arthritis, postmenopausal osteoporosis, Paget's disease, and bone malignancies, such as multiple myeloma (Vega et al. 2007). Among various biomarkers of bone destruction, the investigation of RANKL and OPG in biological analytes of relevance may deliver reliable information on the state of periodontal disease, but may not be able to predict future activity (Buduneli et al. 2011). This paper provides a review of the current literature, elaborating in more detail the findings specifically on the RANKL-OPG system, with regards to periodontal diseases.

Materials and Methods

Search and selection strategy

A literature search was performed using the U.S. National Institutes of Health PubMed database since August 2011, by employing the following search terms: “periodontitis” OR “periodontal disease(s)” AND “RANKL” OR “OPGL” OR “OPG” AND “gene expression” OR “mRNA expression” OR “protein expression” OR “gingival crevicular fluid” OR “saliva” OR “serum” OR “plasma” The search was limited to the English language. After screening of the titles and abstracts, the full text of publications was obtained for the selected articles. Original research papers were considered, including both clinical studies in human populations (cross-sectional or longitudinal) and *in vivo* experimental periodontitis studies in animal models. *In vitro* studies were considered when necessary, for instance, on the description of the biology of the RANKL-OPG system. A further sub-classification of the studies was based on the association of the RANKL-OPG system with active lesions, periodontal microbiota, other markers of bone resorption, diabetes mellitus, smoking, osteoporosis and periodontal treatment.

Review of Current Literature

Cell sources of RANKL and OPG in periodontal tissues

In order to understand the roles of RANKL and OPG in the pathophysiology of periodontal disease, their cell sources need to be identified. The present knowledge on this topic largely derives from *in vitro* or *in vivo* studies. Under physiological conditions, RANKL in the periodontium is expressed by mesenchymal cells, primarily osteoblasts and PDL cells (Kajiya et al. 2010). The expression of RANKL is

of physiological relevance for the adaptation of the periodontium to occlusal forces, during tooth eruption (Wise et al. 2000), or during orthodontic tooth movement (Garlet et al. 2008; Garlet et al. 2007). In the healthy periodontium, OPG is continually produced by resident periodontal connective tissue fibroblasts and potentially endothelial cells (Kobayashi-Sakamoto et al. 2004), but not epithelial cells (Sakata et al. 1999), or T-cells (Belibasakis et al. 2011c; Kawai et al. 2006).

The primary source of RANKL in periodontal disease is Th1 or Th17 cells, as well as B-cells (Han et al. 2006; Han et al. 2009; Kawai et al. 2006; Vernal et al. 2006). Nevertheless, all resident mesenchymal cells may also express RANKL under bacterial challenge (Belibasakis et al. 2007; Kajiya et al. 2010). Interestingly, T-regulatory cells can attenuate RANKL expression by other activated T-cells (Ernst et al. 2007). OPG production in disease presumably derives by the same cells as in health.

Localization and expression of RANKL and OPG in gingival tissues

An early indication that RANKL and OPG could be of relevance to the periodontium came from *in vitro* work demonstrating that periodontal ligament cells do produce RANKL and OPG and they can as well support osteoclastogenesis through RANKL signalling (Kanzaki et al. 2001). Early *in vivo* studies also demonstrated a functional role of RANKL-producing activated T-cells in *Aggregatibacter actinomycetemcomitans* periodontitis models (Taubman et al. 2001; Teng 2002; Teng et al. 2000).

Clearer indications of the involvement of the RANKL-OPG system in periodontal disease came with studies investigating the expression and localization of these molecules in healthy and diseased human gingival tissue. Immunohistochemical

studies demonstrated significantly higher RANKL and lower OPG staining in periodontitis-affected tissue, compared to healthy gingival tissue (Crotti et al. 2003). Positive staining for RANKL protein was associated primarily with CD3+ lymphocytes (approximately 30% of these cells), whereas weaker staining was detected in the extracellular connective tissue. Fewer CD3+ cells were associated with non-periodontitis tissues. OPG staining was associated with endothelial cells, in both health and disease (Crotti et al. 2003). RANKL-positive lymphocytes are more densely distributed in the inflammatory connective tissue zone of diseased gingival tissue (Lu et al. 2006). Accordingly, *in situ* hybridization studies show that high levels of RANKL-specific mRNA transcripts are localized in inflammatory cells, mainly lymphocytes (Liu et al. 2003).

These findings are further backed by gene expression studies using quantitative real time Polymerase Chain Reaction (qPCR) to investigate RANKL and OPG transcripts in human gingival tissue biopsies (Bostanci et al. 2007b; Garlet et al. 2004; Honda et al. 2006). The rate of detection for RANKL expression is reported at 0-40% in health, 25-80% in gingivitis, 54-100% in Chronic Periodontitis (CP) and 75-100% in Aggressive Periodontitis (AP), whereas for OPG these rates are 70-100% in health and 100 % in all three forms of the disease (Belibasakis et al. 2011a; Bostanci et al. 2007b; Garlet et al. 2004). In a study employing semi-quantitative reverse-transcription PCR, the detection rates of RANKL and OPG are reported to be 100% in both health and CP (Wara-aswapati et al. 2007).

With regards to expression levels, RANKL is reportedly 2.7-15.8-fold higher in CP than in health, when detectable (Belibasakis et al. 2011a; Cesar-Neto et al. 2007; Wara-aswapati et al. 2007), and 1.7-3.0-fold higher in CP than gingivitis (Belibasakis et al. 2011a; Bostanci et al. 2007b; Honda et al. 2006). However, the

comparison of RANKL expression levels between CP and AP yielded conflicting results, ranging from no differences (Garlet et al. 2004) to 10-fold higher expression in AP (Bostanci et al. 2007b). Accordingly, OPG expression was shown to be either higher in CP than in AP (Garlet et al. 2004), or 16-fold lower in CP, than AP (Bostanci et al. 2007b). In comparison to health, lower OPG expression levels were reported in CP, at a range of 0.2-16-fold (Bostanci et al. 2007b; Cesar-Neto et al. 2007; Wara-aswapati et al. 2007). Such discrepancies may reflect the episodic nature of periodontitis and highlight the technical challenge of sampling while capturing ongoing disease activity.

Collectively, these findings denote higher RANKL and lower OPG expression levels in periodontitis, compared to health or gingivitis, in line with the established biological role of these molecules in bone resorption. Two of the studies have also taken into account the RANKL/OPG ratio, demonstrating a 2.2-fold increase in CP compared to health (detectable levels) (Wara-aswapati et al. 2007) or equally elevated levels in CP and AP, compared to health or gingivitis (Bostanci et al. 2007b). These findings warrant further investigation of this measure as a potential biomolecular marker of periodontitis.

Detection and levels of RANKL and OPG in gingival crevicular fluid (GCF)

Studies on gene expression and tissue localization of RANKL and OPG provided tangible evidence that RANKL and OPG are involved in periodontal health and disease. Nevertheless, despite their experimental value, such approaches cannot feasibly assist the diagnosis of periodontal status due to their invasive nature (i.e. a tissue biopsy is required). A promising auxiliary diagnostic approach in Clinical Periodontology is the non-invasive sampling of GCF, the window to the periodontium

(Uitto 2003). A number of pro-inflammatory markers have been detected in this inflammatory exudate (Lamster et al. 2007), which would provide a rational basis for investigating RANKL and OPG as well. Indeed, Mogi and co-workers were the first to demonstrate that RANKL and OPG are detectable in GCF by conventional enzyme-linked immunosorbent assay (ELISA) (Mogi et al. 2004). RANKL was detected in only 1/28 healthy sites, but was readily detectable in CP, irrespective of the severity. In fact, it appears that RANKL concentrations were higher when the severity was milder (ap. 180 pg/ μ l), but decreased at a moderate or severe stage (ap. 30 pg/ μ l). The reverse was the case for OPG, which was detected at higher levels in health (ap. 400 pg/ μ l), but was 3-4-fold lower in CP, irrespective of disease severity. Accordingly, the RANKL/OPG ratio was elevated in CP compared to health, while this also proved to be 50% higher in mild, compared to moderate or severe disease (Mogi et al. 2004). These findings were followed up in further studies. The frequency of detection of RANKL in health and CP, was reported at 33% and 100% (Bostanci et al. 2007a), 46% and 85% (Vernal et al. 2004), and 93% and 100% (Lu et al. 2006), respectively. In all cases, the RANKL concentrations or total amounts were higher in CP than in health. OPG was detected at a frequency of 100% in both healthy and CP samples (Bostanci et al. 2007a). However, another study reports OPG detection frequency of 0% in healthy sites of healthy subjects, 75% in healthy sites of subjects with CP, and 72% in diseased sites of patients with CP (Lu et al. 2006). It is not clear if such discrepancies account for intra-individual differences between study populations, or are a result of methodological variations. In gingivitis, a low frequency of RANKL detection was revealed (41%), with concentration levels similar to health (below 10 pg/ μ l) (Bostanci et al. 2007a). When the RANKL/OPG ratio was considered, a clear increase is detected in periodontitis compared to health or

gingivitis, but no differences are detected between CP and AP, narrowing down to the conclusion that the RANKL/OPG ratio could be a good indicator of the occurrence of periodontitis (Kinane et al. 2011; Taubman et al. 2007). An analytical summary of various studies investigating RANKL and OPG concentrations in GCF, as well as their relative ratio in health, gingivitis, CP and AP is provided in the Table. It should be noted, that the available studies do not take under consideration the possibility that RANKL may exist in different isoforms, and rarely consider that it may occur in a molecular complex with OPG. In the available methodologies (primarily ELISA), the detection antibodies may not necessarily be suitable for the detection of all three RANKL isoforms, making difficult the evaluation of their physiological or pathological relevance in GCF.

A more complex point that warrants further investigation is the association of RANKL and OPG to clinical measures of periodontal disease. There are discrepancies between studies, indicating that RANKL or the RANKL/OPG ratio correlates negatively (Mogi et al. 2004), positively (Bostanci et al. 2007a; Vernal et al. 2004) or not at all (Lu et al. 2006) with disease severity. Still, there appears to be no correlation between RANKL/OPG ratio in GCF and gingival inflammation (Bostanci et al. 2007a; Mogi et al. 2004), which may indicate that this measure shows some specificity for the occurrence of periodontal bone destruction, hence periodontitis, rather than constituting a “conventional” or “classical” marker of periodontal inflammation.

Association of RANKL and OPG with active periodontal lesions

A rather obscure issue is the association of the RANKL-OPG system with disease activity at different periodontal sites, which may explain the previously mentioned

discrepancies between clinical measurements and the RANKL/OPG ratio. Most of the studies in the field are of cross-sectional nature and have not taken under consideration the disease progression status of the periodontal sites. Progressive tissue destruction can be assessed by the tolerance method, whereby active sites are determined as the ones that exhibit attachment loss > 2.0 mm during the following 2-month period (Haffajee et al. 1983). The few studies that employed this approach indicate that active CP sites exhibit higher RANKL tissue expression by 3.4-fold (Dutzan et al. 2009) and GCF concentrations by 18% (Silva et al. 2008; Vernal et al. 2004), than inactive ones. The collective conclusion from these studies is that episodic attachment loss is associated with higher RANKL levels than disease remission. However, these studies have not considered the investigation of OPG, which would have allowed for a broader monitoring of the RANKL/OPG ratio during active periodontal disease. Hence, conclusions on the association of the RANKL-OPG system with disease activity may still be preliminary.

RANKL and OPG levels in saliva

A number of studies have investigated the presence and levels of RANKL and OPG in whole un-stimulated saliva, in an attempt to determine their potential use as salivary biomarkers of periodontal disease. In one of the first studies, salivary RANKL concentration was below detection limits (0.375 pmol/L) in 81% of the subjects irrespective of their healthy or CP status, whereas TNF- α was detected in all subjects was significantly higher in subjects with CP (4.33 pg/ml), compared to healthy ones (2.03 pg/ml) (Frodge et al. 2008). Other studies in saliva detected RANKL concentration at a range of 20-200 pg/ml (Buduneli et al. 2008), or 60-110 pg/ml (El-Sharkawy et al. 2010), and OPG at a range of 40-250 pg/ml (Buduneli et al. 2008), in

various subject groups. Still, the available literature is rather limited to deduce secure conclusions on the diagnostic value of RANKL and OPG in saliva.

Association of RANKL and OPG with periodontal microbiota

As oral bacteria are the etiological factor of periodontal diseases, it is rational to postulate that putative periodontal pathogens would be important determinants of RANKL and OPG regulation in periodontal tissues. To this extent, species associated with subgingival biofilms can more potently up-regulate the RANKL/OPG ratio, compared to supragingival biofilms *in vitro* (Belibasakis et al. 2011b). Clinically, the presence of *Porphyromonas gingivalis* in subgingival biofilms at CP sites, positively correlates ($r = 0.64$) with RANKL tissue gene expression levels or the RANKL/OPG ratio ($r = 0.67$), but not with OPG ($r = -0.24$) (Wara-aswapati et al. 2007). Accordingly, a positive correlation is found between RANKL total amounts in GCF and the subgingival presence of *P. gingivalis* and *Treponema denticola* in CP ($p < 0.05$, no r correlation coefficient reported), but no microbial correlations are found in healthy individuals (Sakellari et al. 2008). The association between microbiota and the RANKL/OPG ratio certainly warrants further investigation, to determine if specific bacterial species are more highly implicated in periodontal bone destruction than others. This may enable the design of more targeted therapeutic approaches, aiming at microbial-specific reduction or elimination.

Association of RANKL and OPG with other markers of bone resorption

The activation of osteoclast precursors by RANKL through RANK receptor triggers several intracellular events that eventually lead to their differentiation into multinucleated osteoclasts. Nuclear Factor of Activated T-cells, cytoplasmic 1 (NFATc1) is

the master transcription factor for osteoclastogenesis down-stream the RANKL-RANK signalling, responsible for activating most genes of osteoclast-specific function (Takayanagi 2007; Takayanagi et al. 2002). NFATc1 gingival tissue gene expression is higher in periodontitis, compared to gingivitis or health, showing a strong positive correlation ($r = 0.846$) with RANKL expression (Belibasakis et al. 2011a). Although this finding may potentially denote on-going osteoclast differentiation in the periodontium, it is also possible that NFATc1 expression accounts for RANKL-expressing activated T-cells. To this extent, the expression of dendritic cell-specific transmembrane protein (DC-STAMP), which is involved in osteoclast fusion and multi-nucleation, was also investigated. It was found that DC-STAMP was only sparsely detected in these tissues (0% in health, 20% in gingivitis, 11% in CP and 15% in AP), which may denote a limited number of osteoclast fusion events at the time of sample collection. Hence, the cellular source of NFATc1 in the periodontium awaits further clarification.

Cathepsin-K is a major cysteine proteinase of the osteoclasts, responsible for hydrolysing extracellular bone matrix proteins, upon dissolution of the inorganic matrix by acid secretion (Teitelbaum 2000). Hence, as an end product of osteoclast function, Cathepsin-K is a candidate marker of on-going periodontal bone destruction. To this extent, Cathepsin-K was not detected in GCF from healthy subjects, but was found in increased concentrations in GCF from patients with CP, irrespective of disease severity. A positive correlation ($r = 0.726$) with RANKL concentrations was also revealed (Mogi et al. 2007). This finding could provide clinical justification of the molecular events activated in the osteoclast by RANKL.

Tumor necrosis factor- α -converting enzyme (TACE) is a metalloprotease that can shed and release several cytokines from the cell membrane, thus propagating their

pro-inflammatory or growth effects (Black et al. 1997). Interestingly, compared to several other “sheddas”, TACE has shown the highest specificity for cleaving and releasing RANKL (Lum et al. 1999). TACE total amounts and concentrations were studied in GCF, demonstrating equally higher levels in CP and AP, compared to health or gingivitis, as well as a positive correlation ($r = 0.243$) with RANKL levels (Bostanci et al. 2008). In another study, TACE protein levels were measured in gingival tissues (by Western blot), as well as GCF (by ELISA). Compared to health, a similar trend of elevated TACE levels was observed in both gingival tissue and GCF (respectively: 1.7-fold in gingivitis, 2.6- and 1.8-fold in moderate CP, 2.3- and 1.5-fold in severe CP) (Lee et al. 2011). By studying a group of CP patients who were under treatment with immunosuppressants, it was also deduced that T-cells are the likely source of this enzyme in the diseased periodontium (Bostanci et al. 2008). Reduction of TACE levels or inhibition of its activity may therefore be of interest for future adjunct therapeutic approaches.

Association of RANKL and OPG with smoking

Since smoking is a major risk factor in the development of periodontal disease (Johnson et al. 2007), a number of studies have addressed the additive effect of smoking on the regulation of the RANKL-OPG system, in CP. Interestingly, compared to their matched controls, smokers with CP exhibit lower OPG concentrations in serum (Lappin et al. 2007; Ozcaka et al. 2010), and higher RANKL but lower OPG concentrations in whole saliva (Buduneli et al. 2008), both resulting in enhanced RANKL/OPG ratio. Accordingly, by using qPCR it was found gingival tissue OPG gene expression was 1.4-fold lower, whereas the RANKL/OPG ratio was 1.6-fold higher in smokers than non-smokers with CP (Cesar-Neto et al. 2007). When

the effect of smoking on RANKL and OPG was studied in GCF, there were no differences between former smokers and current smokers with CP. However, OPG concentration and the RANKL/OPG ratio decreased in GCF, when a threshold of 20 pack-years was taken under consideration (Tang et al. 2009). Hence, increased lifetime exposure to smoking could suppress OPG production, resulting in increased RANKL/OPG expression ratio. This may not be surprising given the important histopathological changes of gingival connective tissue in smokers, as the cells of this tissue are the major producers of OPG in the healthy periodontium. Collectively, these findings provide evidence that smoking can indeed affect the RANKL-OPG system in a manner that would further enhance bone loss in periodontitis.

Association of RANKL and OPG with osteoporosis

Osteoporosis is a disease of bone metabolism that could potentially have an impact on the development of periodontal disease (Lerner 2006). *In vivo* studies in experimental (ligature-induced) periodontitis, demonstrated that osteoporotic rats exhibit more severe histopathological tissue breakdown and higher proportions of RANKL-positive cells, than non-osteoporotic ones (Allam et al. 2010). On the clinical aspect, osteoporotic subjects with CP exhibit higher RANKL and OPG plasma concentrations that matched periodontally-healthy subjects (Jabbar et al. 2011), although there appear to be no differences in their relative RANKL/OPG ratio. Nevertheless, further studies with stringent periodontal diagnosis, which would also involve GCF measurements, would shed more light on the interplay between the RANKL-OPG system and osteoporosis, in periodontitis.

Association of RANKL and OPG with diabetes mellitus

Diabetes mellitus is an endocrine disorder that constitutes a major systemic risk factor for periodontal disease (Lamster et al. 2008). Diabetics exhibit higher plasma OPG concentrations and lower RANKL/OPG ratio compared to non-diabetics, and periodontal status does not significantly affect this (Lappin et al. 2009). However, diabetes mellitus appears to be an important factor affecting the RANKL/OPG ratio locally in the periodontium. A recent study using semi-quantitative PCR investigated gingival tissue gene expression in patients with CP, and reported that the diabetic status did not considerably affect RANKL expression, whereas there was a trend towards down-regulation of OPG expression, compared to matched non-diabetics (Duarte et al. 2011). Further studies analysing GCF demonstrate that RANKL concentration and the RANKL/OPG ratio are higher in poorly-controlled diabetics with CP, than in well controlled diabetics, or non-diabetics, with matched periodontal status (Ribeiro et al. 2011; Santos et al. 2010), and this ratio is barely affected by periodontal therapy (Santos et al. 2010). Hence, it appears that the RANKL/OPG ratio is negatively influenced in diabetes mellitus, particularly by poor glycemic control.

Effect of periodontal treatment on the RANKL-OPG system

The role of the RANKL-OPG system is therefore well documented in periodontal disease, reflected by an increased RANKL/OPG ratio, which could be a good indicator of molecular diagnostic value for the disease. However, the remaining question is if periodontal treatment can in fact reduce this ratio to the levels of health, and thus provide a good predictive value for the treatment of the disease. The few available studies employing the initial non-surgical phase of periodontal therapy (including oral hygiene instructions and scaling and root planing) demonstrate that

this may not be the case. Gingival tissue RANKL and OPG gene expressions were evaluated by semi-quantitative PCR, in a cohort of CP subjects that received periodontal surgery 4-6 weeks after the initial phase of periodontal treatment. It was found that both genes were expressed at lower levels than in tissues from healthy control sites (undergoing crown-lengthening procedures), but with no significant differences in the RANKL/OPG ratio (Dereka et al. 2010). Despite the inclusion of healthy controls in that study, it is difficult to drive conclusions on the effect of initial periodontal treatment from the reported data, as samples could not be taken from the actual diseased sites in advance of the therapy. This highlights the ethical and technical limitations of invasive tissue sampling in prospective studies.

Further on, studies have investigated the effect of periodontal treatment on the RANKL-OPG system in GCF. In an earlier study, the analysis of GCF following initial periodontal treatment indicated no changes in RANKL but a decrease in OPG, with a potential increase in the RANKL/OPG ratio after 4 weeks. In addition, no differential responses were observed between smokers and non-smokers (Buduneli et al. 2009). Accordingly, initial periodontal treatment caused a transient reduction of the RANKL/OPG ratio in well controlled diabetics, compared to baseline or poorly controlled diabetics, which, however, resumed baseline levels after 6 months of treatment (Santos et al. 2010). In a recent study employing a cohort of CP and AP patients, initial periodontal treatment did not affect the RANKL/OPG ratio in either group up-to 4 months of monitoring, despite the improved clinical outcome (Bostanci et al. 2011).

Collectively, these studies indicate that despite its potential value as a biomarker for untreated periodontitis, the RANKL/OPG ratio may not be a suitable predictor of clinically successful treatment outcome. It may also denote that the

molecular mechanisms of bone resorption are still active, and thus the corresponding periodontal sites can be potentially at a risk of further disease relapse.

Effects of experimental adjunct periodontal treatment modalities on the RANKL-OPG system

The increased RANKL/OPG ratio following initial periodontal therapy may warrant additional adjunctive treatment modalities. Some of them have been tested in experimental periodontitis models, as for their improved efficiency in clinical outcomes. The inhibition-by-OPG approach has been used in one of the first *in vivo* studies to demonstrate the role of the RANKL-OPG system in experimental periodontitis (Teng et al. 2000). In this T-cell specific *A. actinomycetemcomitans*-induced periodontitis model in mice, concomitant administration OPG diminished alveolar bone destruction and reduced osteoclast numbers in the periodontium. In a later study using a ligature-induced periodontitis model in rats, subcutaneous co-administration of OPG-tagged-Fc resulted in preserved alveolar bone volume and reduced osteoclast number on the alveolar crest surface (Jin et al. 2007). These findings imply that the “natural” inhibition of RANKL by OPG could confer a therapeutic benefit in the prevention of alveolar bone resorption in periodontitis.

The effects of an anti-RANKL antibody on periodontal bone resorption were tested *in vivo* in a T-cell specific *A. actinomycetemcomitans*-induced periodontitis model. Local (palatal) co-administration of a rabbit anti-RANKL IgG, resulted in inhibition of bone resorption and osteoclast formation on the alveolar bone surface, and decreased RANKL concentrations in gingiva, proposing an immunological approach to ameliorate periodontitis (Lin et al. 2011).

In an earlier study using a similar *A. actinomycetemcomitans*-induced periodontitis model, the potassium channel blocker kaliotoxin was systemically administered to rats. This abrogated bone resorption by 84 %, along with a decrease in RANKL mRNA expression by T-cells in the gingival tissue, proposing an interventional strategy for prevention or inhibition of alveolar bone loss (Valverde et al. 2004).

In a recent clinical study, a dietary approach was employed: CP patients received initial periodontal treatment and a combination of omega-3 fatty acids and aspirin, or placebo, for up-to 6 months. The test group exhibited a reduction of RANKL concentrations in saliva and a greater improvement of clinical parameters, compared to the placebo group, proposing a sustainable, low-cost intervention to augment periodontal therapy (El-Sharkawy et al. 2010).

Discussion and Conclusion

All the available studies collectively indicate that both RANKL and OPG can be detected in gingival tissue and biological fluids, including GCF, saliva and serum. RANKL is increased whereas OPG is decreased in periodontitis compared to health, or gingivitis. These regulations are well represented by the increased RANKL/OPG ratio, indicative of the occurrence of periodontitis. However, the RANKL/OPG ratio is not reduced or “switched-off” following a clinically successful initial periodontal treatment outcome. Therefore, this ratio may have a potential value as a biomarker for untreated periodontitis, or denote prior history of the disease at the site level, but it may not be a suitable predictor of clinically successful treatment outcome. Importantly, an increased RANKL/OPG ratio may also denote that the molecular mechanisms of bone resorption are still active, and that the corresponding periodontal

sites are still at a potential risk of further relapse at a future time-point. This flags-up the need for consideration of additional (immuno-) modulatory therapies targeting the RANKL-OPG-specific host responses in the long-term management of periodontal diseases, in line with the concept of Taubman and co-workers (Taubman et al. 2007).

So how could we use the RANKL/OPG ratio further? What do we need to do in order to more efficiently define if it can be of predictive value for on-going disease activity, or an indicator of higher risk for further disease progression? Rather than the available cross-sectional studies, longitudinal prospective studies will be required, in order to monitor concomitantly clinical disease progression and the levels of the RANKL/OPG ratio. Interventional studies will also be needed, utilizing adjunctive treatments and monitoring again changes in this ratio, to determine if its reduction is commensurate with longer term maintenance of successful clinical outcome.

Last but not least, it becomes apparent through the current periodontal literature that there is considerable variation between studies in the reported levels of RANKL, OPG or RANKL/OPG ratio levels (Table). Although there is as yet no stringent definition of the pathological concentrations of these measures in biological fluids, an early study in systemically healthy adults identified that the RANKL/OPG ratio in serum was 2.18 in men and 2.78 in women (Jung et al. 2002). However, at present there is no consensus in such values with regards to healthy and diseased periodontium. The need is raised to establish universal methodological and technical standards for the study of the RANKL-OPG system in the periodontium. In other terms, there is a requirement for globally standardised sample collection techniques and detection assays. The upper or lower limits of physiological RANKL and OPG concentrations in GCF, saliva and serum need to be defined and a "healthy-range" or "periodontitis-range" RANKL/OPG ratio should be deduced.

Clinical Relevance

Scientific rationale for study:

RANKL induces bone resorption, whereas OPG inhibits this action. This Review summarizes the current knowledge on the RANKL-OPG system in relation to Clinical Periodontology, discussing its diagnostic and prognostic value for periodontal diseases.

Principal findings:

Periodontitis is associated with increased RANKL and decreased OPG levels in gingival tissue and GCF, resulting in increased RANKL/OPG ratio. Smoking and diabetes mellitus further enhance this ratio. Conventional periodontal treatment may not affect this ratio.

Practical implications:

Although the RANKL/OPG ratio may have a potential as a biomarker for periodontitis, its predictive value for the on-going disease is unclear. Further adjunct treatment modalities may be required to decrease this ratio.

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Table: Studies involving GCF concentrations of RANKL and OPG, and RANKL/OPG ratio levels in health and periodontal disease.

Publication	Study popul.	RANKL (pg/μl)	OPG (pg/μl)	Ratio
Mogi <i>et al</i> 2004	CP (n=132)	ap. 30-180	ap. 50-110	ap. 1.0-1.7
	Health (n=28)	ap. 30	ap. 350-450	ap. 0.1
Vernal <i>et al</i> 2004	CP (n=20)	116.99 ± 83.04	N/A	N/A
	Health (n=12)	188.07 ± 103.40	N/A	N/A
Lu <i>et al</i> 2006 *	CP (n=20)	0 - 285.75	0-29.05	N/A
	Health (n=4)	0 - 45.16	9.67 - 40.7	N/A
Bostanci <i>et al</i> 2007	CP (n=28)	370.7 ± 54.7	119.6 ± 14.4	3.19 ± 0.23
	AP (n=25)	387.5 ± 42.8	101.5± 10.2	3.81 ± 0.15
	Ging. (n=22)	9 ± 5.7	167.6 ± 20.6	0.05 ± 0.03
	Health (n=21)	6.8 ± 3.8	408.1 ± 36.6	0.017±0.008
Mogi, Ootogoto 2007	CP (n=66)	87.7 ± 16.2	N/A	N/A
	Health (n=19)	10.9 ± 2.8	N/A	N/A
Sakellari <i>et al</i> 2008 *	CP (n=35)	0.19 ± 0.04	N/A	N/A
	Health (n=38)	0.07 ± 0.17	N/A	N/A
Silva <i>et al</i> 2008	CP (n=56)			
	Active sites:	131.17 ± 35.1	N/A	N/A
	Inactive sites:	108.80 ± 18.9	N/A	N/A
Buduneli <i>et al</i> 2009	CP (n=20)			
	Smokers:	0.44 ± 0.27	1.78 ± 1.9	0.25

	<i>Non-smokers</i>	0.60 ± 0.46	2.40 ± 4.26	0.25
Santos <i>et al</i> 2010	CP (n=38)			
	<i>Well controlled diabetics:</i>			
		ap. 100	ap. 40	ap. 2.5
	<i>Poorly controlled diabetics:</i>			
		ap. 250	ap. 25	ap. 10
Ribeiro <i>et al</i> 2011	CP (n=20)	86.2 ± 39.9	13.4 ± 12.5	12.3 ± 12.7
	CP diabetics (n=37)			
	<i>Well controlled diabetics:</i>			
		222.8 ± 294.2	45.0 ± 66.6	5.3
	<i>Poorly controlled diabetics:</i>			
		402.5 ± 286.5	50.6 ± 61.6	24.7
Bostanci <i>et al</i> 2011	CP (n =14)	433 ± 269	51 ± 29	9.1 ± 3.5
	AP (n =13)	468 ± 580	71 ± 74	5.9 ± 1.4

Table Legend: The Table shows the concentrations of RANKL, OPG and RANKL/OPG ratio in GCF from healthy individuals and patients with gingivitis, CP and AP, analyzed by ELISA in different studies. The studies marked with an asterisk (*) have measured the total amounts of either molecule in GCF (provided as pg/site, for 30 sec sampling), whereas the others have calibrated these values for the measured GCF volume per site (provided as pg/ μ l). Where the actual means or ranges are not provided in the respective papers, an approximation (ap.) of the values is given, based of the available Figures. In papers where periodontal treatment is involved, only the baseline levels are given.

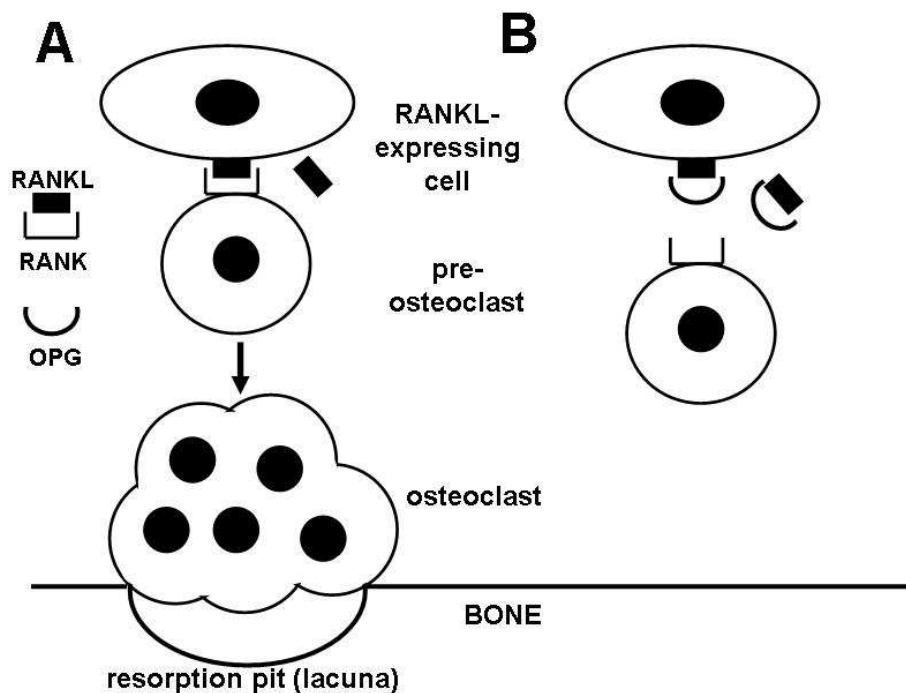


Figure Legend

Schematic representation of the action of the RANKL-OPG system. (A) RANKL is expressed as a membrane bound (or secreted) ligand by a number of cell types, such as osteoblasts, fibroblasts, bone marrow stromal cells, and activated T- and B-cells. When RANKL binds to its cognate RANK receptor on the surface of pre-osteoclasts (cells of the monocyte/ macrophage cell lineage), it triggers a number of intracellular processes that lead to their fusion and differentiation into multi-nucleated mature osteoclasts (process called “osteoclastogenesis”). On the tissue level, osteoclasts attach on the bone surface and subsequently resorb bone. (B) The action of RANKL can be blocked by its soluble decoy receptor OPG, which has structural homology to RANK. When OPG binds to RANKL, it prevents its further interaction with RANK, thus inhibiting all the down-stream molecular events that would lead to osteoclast differentiation and bone resorption. Hence, OPG is an inhibitor of bone resorption.